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Pisolithus: a new species from southeast Asia and a new combination

C. Phosri[1*], M.P. Martín[2], N. Suwannasai[3], P. Sihanonth[4] & R. Watling[5]

[1]*Microbiology programme, Pibulsongkram Rajabhat University, Phitsanulok, 65000, Thailand*
[2]*Departamento de Micologia, Real Jardín Botánico, CSIC, Plaza de Murillo 2, 28014, Madrid, Spain*
[3]*Department of Biology, Srinakharinwirot University, Bangkok, 10110, Thailand*
[4]*Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand*
[5]*Caledonian Mycological Enterprises, Crelah, 26 Blinkbonny Avenue, Edinburgh, EH4 3HU, Scotland*
*Correspondence to: *cherd_phosri@yahoo.co.uk*

Abstract — Based on morphological and ITS nrDNA sequence analyses, a species recognized in classical European literature as *Lycoperdon capsulifer* is transferred to *Pisolithus* and *P. orientalis* is proposed as a new species. *Pisolithus orientalis* basidiomes, collected under *Pinus kesiya* in Thailand, correspond to *Pisolithus* sp. 5 sensu Martin et al. *Pisolithus* sp. 4 sensu Martin et al. is shown to comprise two groups, one equivalent to *P. arhizus* as collected from *Pinus* and *Quercus* forests from Italy and Spain and the other represented by the new *P. capsulifer* from mixed pine-deciduous forests in England and France.

Key words — ectomycorrhizal fungi, gasteromycete, phylogenetic species, PT, taxonomy

Introduction

Pisolithus is one of the most widespread and cosmopolitan gasteromycete genera ranging from temperate to tropical regions of the world. It has long been considered as monotypic (Coker & Couch 1928, Cunningham 1942, Pilát 1958) and is well-researched, primarily based on ectomycorrhizal studies, as *Pisolithus tinctorius* (P. Micheli ex Pers.) Coker & Couch (Marx et al. 1984, Burgess et al. 1995). Rauschert (1959) proposed that this species was conspecific with *Polysaccum arhizum* Scop., which he recombined as *Pisolithus arhizus* (Scop.) Rauschert. However, after molecular studies indicated that *P. tinctorius* is a species complex (Anderson et al. 1998, Cairney et al. 1999, Díez et al. 2001), Martin et al. (2002) proposed at least 11 phylogenetic species. Several subsequent morphological and molecular studies have confirmed

Table 1. *Pisolithus* specimens sequenced in the present study. * = Genbank sequences obtained from cultures.

Taxon	DNA code	Herbarium No.	Origin	Potential host	Collection date	Genbank No.
P. abditus (sp. 12)	24PISOLI	BBH28595	Roi Et, Thailand	*Shorea roxburghii*	08-Jul-2001	FR748119
	31PISOLI	BBH28596	Yasothon, Thailand	*S. roxburghii*	13-Jun-2002	FR748120
P. albus (sp. 7)	12PISOLI	—	Yasothon Thailand	*Eucalyptus camaldulensis*	—	AJ629887*
	13PISOLI	—	Petchaboon, Thailand	*E. camaldulensis*	29-Jan-2002	FR748121*
	14PISOLI	—	Chaiyapum, Thailand	*E. camaldulensis*	28-Jan-2002	FR748122*
	15PISOLI	—	Lumpoon, Thailand	*E. camaldulensis*	28-Jan-2002	FR748123*
	17PISOLI	—	Nakornsawan, Thailand	*E. camaldulensis*	28-Jan-2002	FR748124*
	20PISOLI	BBH28593	Waimea, Kona, HI, USA	*Eucalyptus* spp.	2002	FR748125
	21PISOLI	BBH28594	Australia	*Eucalyptus* spp.	2002	FR748126
	35PISOLI	BBH28599	Narathiwas, Thailand	*Melaleuca* spp.	2002	FR748127
P. arhizus (sp. 4)	3PISOLI	BCN-MPM2676	Tarragona, Spain	*Pinus* sp.	Oct-1997	FM213365
	92bisPISOLI	Wat.139161	Sardinia, Italy	—	Apr-2000	FR748128–FR748132
	6PISOLI	BCN-MPM2597	Tarragona, Spain	*Pinus* sp.	27-Nov-1996	FR748133
P. capsulifer (sp. 14)	51PISOLI	E00159834	Berkshire, England	—	Sep-1970	FR748134
	46PISOLI	E00290168 (**epitype**)	Berkshire, England	—	07-Sep-1993	FR748135
	49PISOLI	E00185017	Berkshire, England	*Pinus sylvestris, P. contorta*	09-Sep-1973	FR748136
	53PISOLI	E00159822	Limoges, France	Mixed broadleaf & *Pinus* spp.	01-Sep-1993	FR748137
P. tinctorius (sp. 6)	5PISOLI	BCN-MPM2596	Alt Empordà, Girona, Spain	*Pinus* sp.	22-Jan-1995	FR748138
	19PISOLI	BBH28592	Juneau County, WI, USA.	*Quercus* & *Pinus* spp.	21-Sep-2001	FR748139
Pisolithus sp. 3	ARHPIS3	MJ36192	Huelva, Spain	*Cistus* sp.	01-Jan-2003	FR748140–FR748142
P. orientalis (sp. 5)	7PISOLI	—	Chiang Mai, Thailand	*Pinus kesiya*	11-Aug-2001	FR748143*
	8PISOLI	—	Chiang Mai, Thailand	*P. kesiya*	11-Aug-2001	FR748144*
	9PISOLI	—	Petchaboon, Thailand	*P. kesiya*	28-Jan-2002	FR748145*
	10PISOLI	—	Petchaboon, Thailand	*P. kesiya*	28-Jan-2002	FR748146*
	11PISOLI	—	Petchaboon, Thailand	*P. kesiya*	28-Jan-2002	FR748147*
	33PISOLI	BBH28597 (**holotype**)	Chiang Mai, Thailand	*P. kesiya*	11-Aug-2001	FR748148
	34PISOLI	BBH28598	Chiang Mai, Thailand	*P. kesiya*	11-Aug-2001	FR748149

the taxonomic rank of some old species [*Pisolithus albus* (Cooke & Massee) Priest, *P. marmoratus* (Berk.) E. Fisch., *P. microcarpus* (Cooke & Massee) G. Cunn.] and established several new ones (*Pisolithus abditus* Kanch. et al., *P. aurantioscabrosus* Watling, *P. indicus* Natarajan & Senthil.). Nonetheless, some clades recognised by Martin et al. (2002) remain without specific names.

Additional morphological studies and ITS nrDNA sequence analyses of recently collected austro-asiatic, euro-asiatic, and paleotropical *Pisolithus* specimens have been conducted to assign morphological species to the *Pisolithus* sp. 5 phylogenetic lineage and to members of the AII clade of Martin et al. (2002).

Materials & methods

Fungal isolates

The *Pisolithus* material used in this work included fresh, herbarium, or cultured specimens sampled from a variety of geographical sites (Table 1). Basidiomes for each isolate are retained in the herbarium at the Microbiology Programme, Faculty of Science & Technology, Pibulsongkram Rajabhat University and BBH Thailand. Cultures obtained from basidiomata were grown and maintained on Modified Melin Norkrans' (MMN) medium (Marx & Kenny 1982) at 30°C with subculturing every 1–2 months. Herbarium specimens were provided by BCN and E (Abbreviations for herbaria follow Lanjouw & Stafleu 1964).

Molecular methods

Isolation, amplification, purification, and sequencing of DNA followed Phosri et al. (2007). DNA was isolated using the E.Z.N.A. fungi DNA miniprep kit (Omega Biotech, Doraville, Georgia, USA) as described by Martín & García-Figueres (1999) or a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). ITS regions were amplified with Ready-To-Go™ PCR Beads (Amersham-Pharmacia Biotech, NJ, USA) (Martín & Winka 2000). Primer pairs ITS1F/ITS4B and ITS1/ITS4 were used to amplify the ITS1+5.8S+ITS2 nrDNA gene cluster following White et al. (1990) and Gardes & Bruns (1993). Prior to sequencing, products were cleaned using either E.Z.N.A. Clean kit (Omega Biotech) or directly from the gel using QIAQuick PCR purification kit (QIAGEN, Chatsworth, CA). Both strands were sequenced separately at Secugen S.L. (Madrid, Spain) or Macrogen (South Korea). All samples were sequenced in both directions.

When needed, DNA was cloned using pGEM®-T Easy Vector System II cloning kit (Promega Corporation, Madison, Wisconsin, USA) and purified with QIAPrep Spin Mini prep (QIAGEN). Both strands were sequenced separately using vector specific primers T7 and SP6 (Nag et al. 1988) at Secugen S.L. or Macrogen.

Sequence analysis

Navigator™ Sequence comparison software (Perkin Elmer Applied Biosystem) or Sequencher™ version 4.2 (Gene Codes Corporations, Ann Arbor, Michigan, USA) was used to assemble the consensus sequence from the two strands of the ITS nrDNA of each isolate. Blastn searches with megablast option were used to compare the sequences obtained against the sequences in the National Center of Biotechnology Information

(NCBI) nucleotide database. The new consensus sequences have been lodged in the EMLB-EBI database with accession numbers indicated in Table 1.

Sequences obtained were compared with homologous sequences of *Pisolithus* spp. retrieved from the EMBL Nucleotide Sequence Database with many published in Martin et al. (2002), Kanchanaprayudh et al. (2003a,b), Moyersoen et al. (2003), and Reddy et al. (2005) to represent all their described clades. Prior to 2009 sequences were aligned using the SEQAPP software (Perkin Elmer Applied Biosystem) and thereafter using SeAl v2.0a11 Carbon (Rambaut 2002). The alignment was optimized visually. Alignment gaps were indicated as "-" and ambiguous nucleotides were marked as "N".

Phylogenetic analyses

The alignment was analysed using the programme PAUP 4.0b10 (Swofford 2003) and MrBAYES v3.0 b4 (Huelsenbeck & Ronquist 2001) as described in Tellería et al. (2010). *Scleroderma citrinum* Pers. (FM213344) and *Suillus luteus* (L.) Roussel (GU373495) were included as outgroups. First, a maximum parsimony analysis (MP) was inferred using the heuristic search option in PAUP. Gaps were treated as missing data. Branch lengths equal to zero were collapsed to polytomies. Nonparametric bootstrap (BP) support (Felsenstein 1985) for each clade was tested based on 10000 replicates, using the fast-step option.

A second analysis was conducted using a Bayesian approach (Larget & Simon 1999, Huelsenbeck & Ronquist 2001). It was performed assuming the HKY + G model as suggested by hierarchical likelihood ratio test (hKRTs) and akaike information criterion (AIC) in MrModeltest 2.3 (Posada & Crandall 1998, Nylander 2004).

A combination of both posterior probabilities and bootstrap proportion was used to assess the level of confidence for a specific node (Lutzoni et al. 2004). The alignment matrix and the 50% majority–rule consensus tree from the Bayesian analysis are available in TreeBase (http://www.treebase.org/).

Recovered lineages and clades are named according to Martin et al. (2002). When a specific name has been formally assigned to a clade, the name is included along with the species number of Martin et al. (2002).

Results

Thirty-three new *Pisolithus* sequences have been generated, including those obtained from the epitype (E00290168) of the new combination, *P. capsulifer*, and the holotype (BBH28597) of the new species, *P. orientalis.* These were aligned with 67 additional sequences obtained from databases (GenBank and UNITE; see Figs 1–2) and phylogenetically analyzed.

The ITS nrDNA dataset contains 97 sequences and 893 aligned positions, of which 410 were constant, 177 parsimony uninformative, and 306 parsimony-informative. Maximum parsimony analysis yielded 100 most parsimonious trees (940 steps long, CI = 0.6681, HI = 0.3319, RI = 0.8968). One best tree found by branch swapping is shown in Fig. 1, with bootstrap values on the branches. The 50% majority-rule consensus tree from the Bayesian analyses is in Fig. 2, with posterior probabilities indicated on the branches.

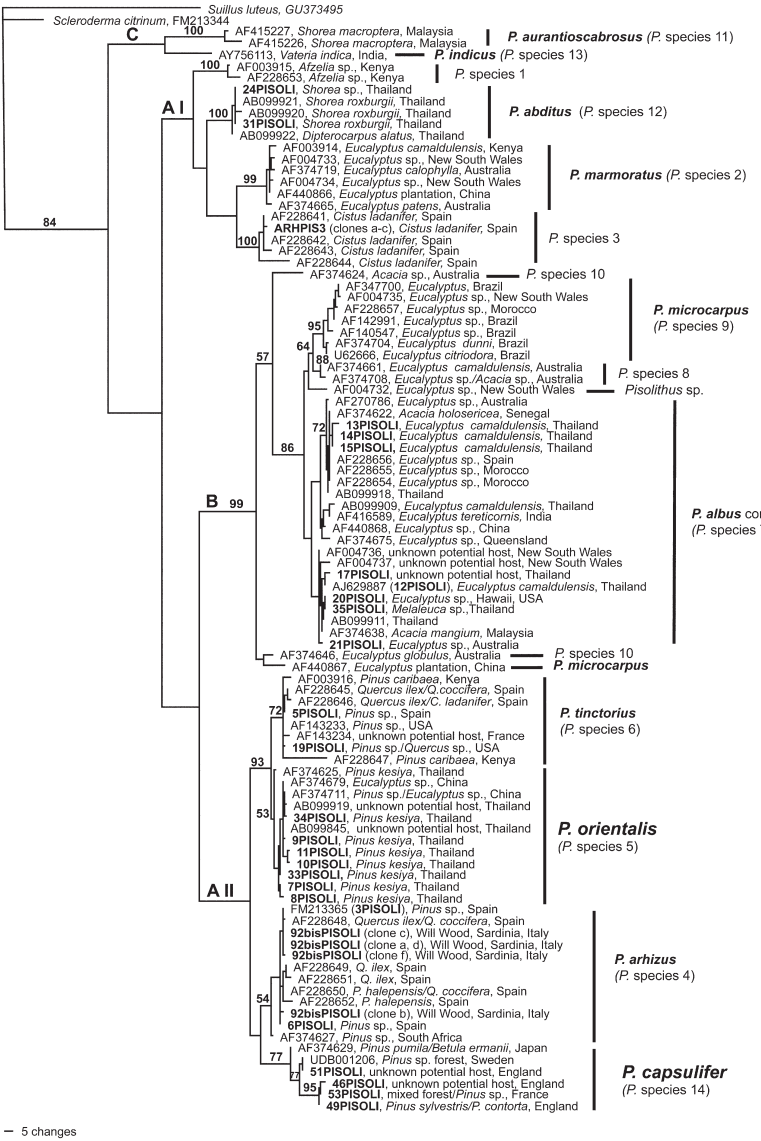
Suillus luteus, GU373495
Scleroderma citrinum, FM213344
C
AF415227, Shorea macroptera, Malaysia
AF415226, Shorea macroptera, Malaysia
P. aurantioscabrosus (P. species 11)
AY756113, Vateria indica, India,
P. indicus (P. species 13)
AF003915, Afzelia sp., Kenya
AF228653, Afzelia sp., Kenya
P. species 1
A I
24PISOLI, Shorea sp., Thailand
AB099921, Shorea roxburgii, Thailand
AB099920, Shorea roxburgii, Thailand
31PISOLI, Shorea roxburgii, Thailand
AB099922, Dipterocarpus alatus, Thailand
P. abditus (P. species 12)
AF003914, Eucalyptus camaldulensis, Kenya
AF004733, Eucalyptus sp., New South Wales
AF374719, Eucalyptus calophylla, Australia
AF004734, Eucalyptus sp., New South Wales
AF440866, Eucalyptus plantation, China
AF374665, Eucalyptus patens, Australia
P. marmoratus (P. species 2)
AF228641, Cistus ladanifer, Spain
ARHPIS3 (clones a-c), Cistus ladanifer, Spain
AF228642, Cistus ladanifer, Spain
AF228643, Cistus ladanifer, Spain
P. species 3
AF228644, Cistus ladanifer, Spain
AF374624, Acacia sp., Australia
P. species 10
AF347700, Eucalyptus, Brazil
AF004735, Eucalyptus sp., New South Wales
AF228657, Eucalyptus sp., Morocco
AF142991, Eucalyptus sp., Brazil
AF140547, Eucalyptus sp., Brazil
AF374704, Eucalyptus dunni, Brazil
U62666, Eucalyptus citriodora, Brazil
P. microcarpus (P. species 9)
AF374661, Eucalyptus camaldulensis, Australia
AF374708, Eucalyptus sp./Acacia sp., Australia
AF004732, Eucalyptus sp., New South Wales
P. species 8
Pisolithus sp.
AF270786, Eucalyptus sp., Australia
AF374622, Acacia holosericea, Senegal
13PISOLI, Eucalyptus camaldulensis, Thailand
14PISOLI, Eucalyptus camaldulensis, Thailand
15PISOLI, Eucalyptus camaldulensis, Thailand
AF228656, Eucalyptus sp., Spain
AF228655, Eucalyptus sp., Morocco
AF228654, Eucalyptus sp., Morocco
AB099918, Thailand
AB099909, Eucalyptus camaldulensis, Thailand
AF416589, Eucalyptus tereticornis, India
AF440868, Eucalyptus sp., China
AF374675, Eucalyptus sp., Queensland
AF004736, unknown potential host, New South Wales
AF004737, unknown potential host, New South Wales
17PISOLI, unknown potential host, Thailand
AJ629887 (12PISOLI), Eucalyptus camaldulensis, Thailand
20PISOLI, Eucalyptus sp., Hawaii, USA
35PISOLI, Melaleuca sp.,Thailand
AB099911, Thailand
AF374638, Acacia mangium, Malaysia
21PISOLI, Eucalyptus sp., Australia
P. albus complex (P. species 7)
B
AF374646, Eucalyptus globulus, Australia
AF440867, Eucalyptus plantation, China
P. species 10
P. microcarpus
AF003916, Pinus caribaea, Kenya
AF228645, Quercus ilex/Q.coccifera, Spain
AF228646, Quercus ilex/C. ladanifer, Spain
5PISOLI, Pinus sp., Spain
AF143233, Pinus sp., USA
AF143234, unknown potential host, France
19PISOLI, Pinus sp./Quercus sp., USA
AF228647, Pinus caribaea, Kenya
P. tinctorius (P. species 6)
AF374625, Pinus kesiya, Thailand
AF374679, Eucalyptus sp., China
AF374711, Pinus sp./Eucalyptus sp., China
AB099919, unknown potential host, Thailand
34PISOLI, Pinus kesiya, Thailand
AB099845, unknown potential host, Thailand
9PISOLI, Pinus kesiya, Thailand
11PISOLI, Pinus kesiya, Thailand
10PISOLI, Pinus kesiya, Thailand
33PISOLI, Pinus kesiya, Thailand
7PISOLI, Pinus kesiya, Thailand
8PISOLI, Pinus kesiya, Thailand
P. orientalis (P. species 5)
A II
FM213365 (3PISOLI), Pinus sp., Spain
AF228648, Quercus ilex/Q. coccifera, Spain
92bisPISOLI (clone c), Will Wood, Sardinia, Italy
92bisPISOLI (clone a, d), Will Wood, Sardinia, Italy
92bisPISOLI (clone f), Will Wood, Sardinia, Italy
AF228649, Q. ilex, Spain
AF228651, Q. ilex, Spain
AF228650, P. halepensis/Q. coccifera, Spain
AF228652, P. halepensis, Spain
92bisPISOLI (clone b), Will Wood, Sardinia, Italy
6PISOLI, Pinus sp., Spain
AF374627, Pinus sp., South Africa
P. arhizus (P. species 4)
AF374629, Pinus pumila/Betula ermanii, Japan
UDB001206, Pinus sp. forest, Sweden
51PISOLI, unknown potential host, England
46PISOLI, unknown potential host, England
53PISOLI, mixed forest/Pinus sp., France
49PISOLI, Pinus sylvestris/P. contorta, England
P. capsulifer (P. species 14)
5 changes

Fig. 1. Phylogenetic tree (one of 100 most parsimonious trees) obtained with a parsimony analysis under heuristic search of ITS sequences of *Pisolithus* collections included in Table 1 and sequences obtained from the GenBank and UNITE. Each branch is labeled with the DNA isolation code in bold (new sequences) or with the accession number including the possible host and the country. Numbers above branches represent bootstrap values. Clades and species number according to previous authors.

In both analyses, *Pisolithus* sequences form a highly supported clade (BS = 84%, PP = 1.0) with at least 15 terminal assemblages. However, the relationships between the clades are generally not well resolved and the main differences between Fig. 1 and Fig. 2 are related to the position of clades from lineage AI in Martin et al. (2002).

The basal clade (BS <50%, PP = 0.62) (lineage C, Martin et al. 2002) included in our study contains *Pisolithus aurantioscabrosus* and *P. indicus,* which are sister to the remaining *Pisolithus* taxa. These two taxa are restricted to native dipterocarp forests in southeast Asia and India, respectively.

Lineage AI is separated into four clades, each with high support (BP ≥ 99% or PP = 1.0). Lineage AII also ramifies into at least four main groups, but these possess weak support (BS < 50%, PP = 0.98). The four AII groups are *Pisolithus* spp. 4, 5, and 6 of Martin et al. (2002), and one group here called *Pisolithus* sp. 14. *Pisolithus* sp. 6 (BS = 72%, PP = 0.99) consisted of eight *Pisolithus* sequences from basidiomata found mainly in association with pine (*Pinus caribaea* Morelet.) and oak (*Quercus ilex* L., *Q. coccifera* L.) in various regions ranging from USA, Spain, Kenya, and France. We consider this clade to be *P. tinctorius* s. st., sister to the *Pisolithus* sp. 5 clade formed by seven new isolates associated with *Pinus kesiya* Royle ex Gordon in Thailand and five GenBank sequences. The unifying features unique to these collections lead us to describe them below as a new species, *Pisolithus orientalis.* The other two clades (*Pisolithus* spp. 4 and 14) in Lineage AII are sister to the clade formed by *Pisolithus tinctorius* s. st. and *P. orientalis.* Collections from the British Isles (46PISOLI, 49PISOLI and 51PISOLI) and France (53PISOLI) clearly separate from *Pisolithus* sp. 4 (BS = 70%, PP = 1.0) and group together with sequences from Sweden (UDB001206) and Japan (AF374629). Sequenced specimens from British Isles, France, and Sweden have been analysed in parallel to basidiospore SEM studies. The electron micrographs (Fig. 3) indicate that the spore ornamentation does not resemble that of any other *Pisolithus* documented. The specific name *Pisolithus capsulifer* has been assigned to *Pisolithus* sp. 14.

Discussion

In lineage AII, one clade ('sp. 14') comprises predominantly British material. *Pisolithus* is very rare in the British Isles, where it is recorded from London, Hampshire, Devon, and Norfolk. Most records are from Tertiary gravels and sandy mineral soils and confined to the southernmost part, especially the southeast of England. There is one record of *Pisolithus* from Ireland 'on a bank in a car park, under *Castanea sativa* and *Pinus* spp., well-drained peat over lying sandy boulder-clay north slope of Knockmealdon Mountains, Kilballyboy Wd., South Tipperary (July 1984)' (Ing 1995), but it is not recorded from Scotland. The British sequences agreed with one each from France and Sweden. Here spore morphology is also distinctive, but is it significant? Grand (1976) and Watling

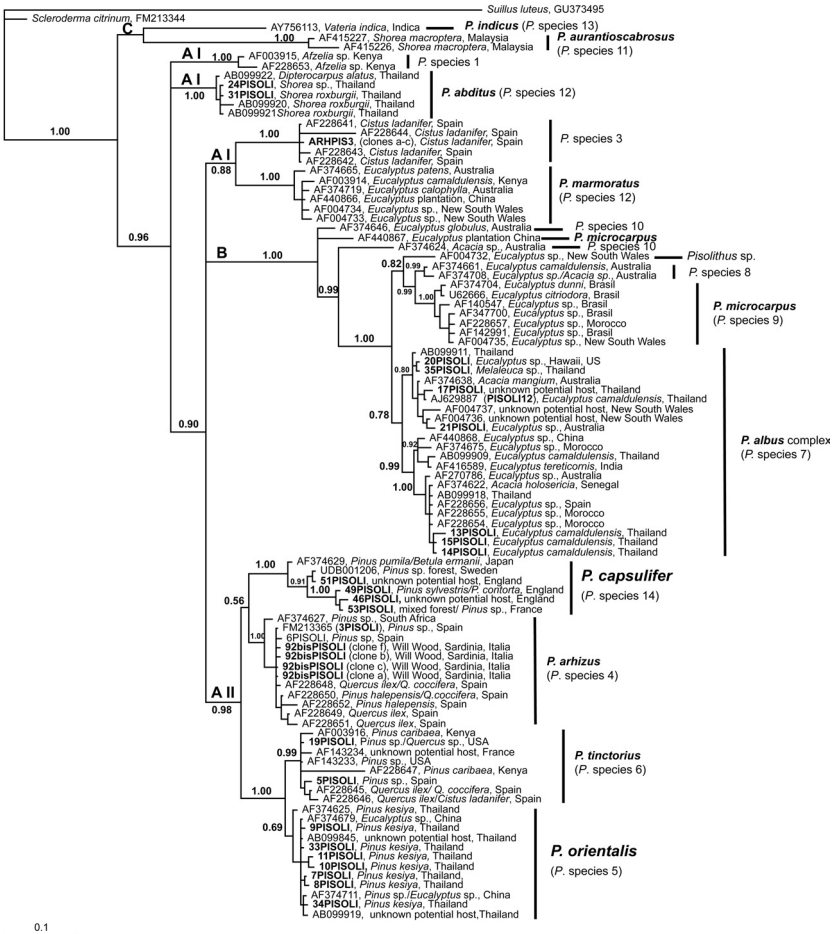

Fig. 2. Phylogenetic tree obtained with a Bayesian Monte Carlo Markov (MCMC) analysis assuming HKY + G model, as suggested by MrModeltest 2.3, of ITS nrDNA sequences of *Pisolithus* collections included in Table 1 and sequences obtained from the GenBank and UNITE. Each branch is labeled with the DNA isolation code in bold (new sequences) or with the accession number including the potential host and the country. Numbers above branches represent posterior probabilities values. Clades and species numbers according to previous authors.

et al. (1999) note that basidiospore morphology should be considered carefully as the mature spore ornamentation relies on spore development in which nurse cells play a vital role and affect final surface features. Morphological differences often have diagnostic importance for new morphological species, (e.g., *Astraeus odoratus;* Phosri et al. 2004, 2007), despite instances of convergence.

Sowerby (1814), who provided an excellent illustration of a gasteroid fungus, apparently coined the epithet '*Lycoperdon capsuliferum*' based on the fact that the fertile part of the fruiting body contained small spore capsules resembling a fig fruit. Fries (1829: 54) recognized the significance of this collection and how it differed from other species known to him mainly from the classical literature. He formally proposed the superfluous new name *Polysaccum olivaceum* in section *Radiculosa* for Sowerby's species, differing from the other members of the section in spore-mass colour and fruiting body shape. Because of the morphological and habitat similarities shared by Sowerby's fungus and modern 'sp. 14' clade collection, we are confident in identifying this material as *Lycoperdon capsuliferum,* which we transfer to *Pisolithus*.

Pisolithus capsulifer (Sowerby) Watling, Phosri & M.P. Martín, **comb. nov.** Fig. 3

MycoBank MB 519949 [*Pisolithus* sp. 14]

≡ *Lycoperdon capsuliferum* Sowerby, Col. Fig. Engl. Fung., Suppl.(no. 31): pl. 425a/b. 1814.

≡ *Polysaccum olivaceum* Fr., Syst. Mycol. 3(1): 54. 1829 [nom. nov. superfl.].

Type: Coloured plate 425a/b in Sowerby, Col. Fig. Engl. Fung., Suppl.(no. 31), 1814 (**lectotype designated here**). ENGLAND, Berkshire, Sandhurst, 7 Sep 1993, leg. E.E. Green, Wat. 25310 (**epitype designated here**, E00290168; GenBank FR748135)

Basidiomes turbinate to pyriform with subglobose to ellipsoid head 40–60 mm diam. or flattened at the apex > 90 mm broad, tapering into solid, firm, irregular pseudostipe incorporating soil debris; pseudostipe 15–30(–90) mm long often flattened and then 10–25 mm broad truncated downwards to 15 mm. Peridium wafer thin, > 1mm thick, dry, smooth, yellowish ochraceous with olivaceous spots and faint netted appearance from outline of pseudoperidioles below, then fragmenting to expose powdery spore–mass and disintegrating pseudoperidioles. Lower part more compacted and with an olivaceous-yellow hue, finally drying hard after weathering to become clinker–like. Pseudoperidioles pale then red–brown finally burnt umber, larger at apex and compressed downwards, becoming pulverulent.

Clamp-connections present. Basidiospores 6.8–*8.2*–10.6 µm, globose, spinose spines at first separate and 1.1–*1.9*–2.9 µm long, before collapsing to coalesce into groups and falling back onto spore surface to give a rivulose effect.

Additional specimens examined: **FRANCE**, 20 km. from Limoges, mixed broadleaf–conifer forest, 1-Sep-1997, leg. E.E. Green, Wat. 25298 (E00159822). **UNITED KINGDOM**, **Hampshire**, Ringwood, 12-Sep-1926, coll. E.J.H. Corner 81444 (E00075037); coll. E.J.H. Corner 81446 (E00075038); **Berkshire**, Windsor Great Park, Jul-1993, leg. E.E. Green, Wat. 25311 (E00290167); Caesar's Camp near Bracknell, Sep-1970, leg. E.E. Green (E00159834); 9–Sep-1973, leg. E.E. Green, Wat. 10118 (E00185017); 3-Oct-1981, leg. E.E. Green, Wat. 14216 (E00185018).

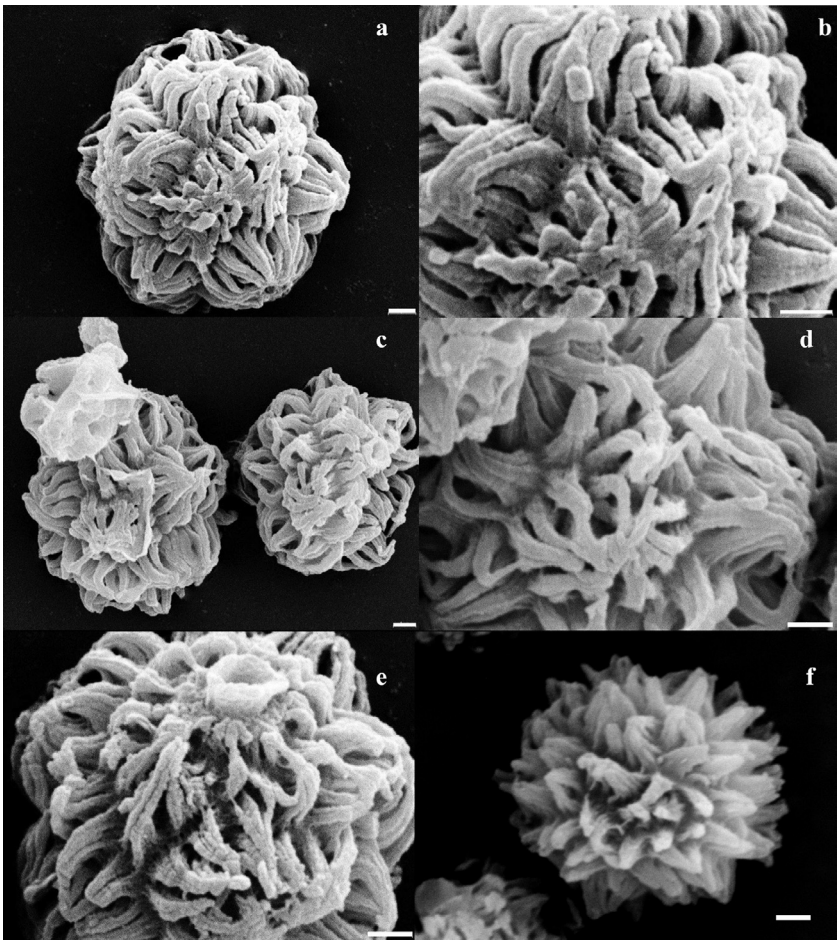

Fig. 3. SEM of spore ornamentation of selected *Pisolithus* collections (bars = 1 µm). a–e: ***P. capsulifer***. From England—a–b: epitype E00290168 (Wat. 25310, 46pisoli), c–d: E00185017 (Wat. 10118, 49pisoli); from France—e: E00159822 (Wat. 25298, 53pisoli). f: ***P. orientalis***. Holotype BBH28597 (33pisoli).

Comments. The distribution of the pseudoperidioles in fresh material is clearly expressed by Sowerby (1814) when he wrote, "at first strong and very rugged but the congeries of seeds, if I may so-call them, were enveloped or compressed into forms laying by each other, giving a reticulated appearance in some directions, being mostly rather oblong, and of a dark brown colour…and looks like pollen bursting from something analogous to anthers."

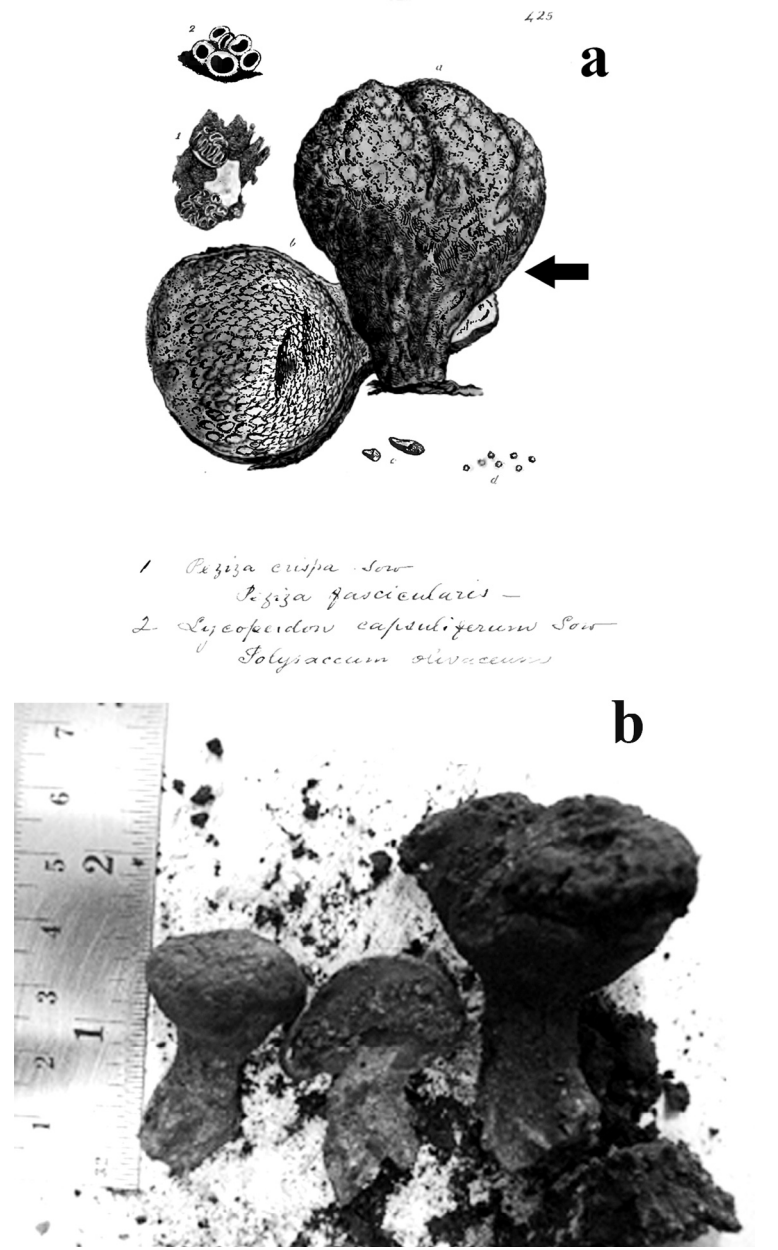

Fig. 4. a: Sowerby's illustration of *Lycoperdon capsuliferum* (arrow).
b: *Pisolithus orientalis* Holotype BBH28597 (33pisoli).

Pisolithus orientalis Watling, Phosri & M.P. Martín **sp. nov.** Fig. 4

MycoBank MB519952 [*Pisolithus* sp. 5]

Differs from *Pisolithus tinctorius* by smooth pedicellate basidiomata with blackish brown discs, smaller globose basidiospores ornamented by isolated groups of narrow cones that adhere together to form secondary spines, and an association with *Pinus kesiya*.

Type: THAILAND. Chang Mai, Hot District, Bo Kaew pine plantation, under *Pinus kesiya*, 11-Aug-2001 (**holotype**, BBH 28597; GenBank FR748148).

Basidiome: 35–55 mm high, pedicellate to broadly clavate, divided into distinct head and cylindrical stipe, snuff-brown to cigar-brown with a black shining surface around the head, surface smooth, not scaly. Peridium: 24–45 mm broad, broadly ellipsoid to subglobose, rough, thin, brittle, snuff-brown, breaking up at the apex to expose powdery contents and join up to stipe. Stipe: 13–24 × 10–15 mm woody, snuff-brown, smooth, cylindrical base of agglutinate mycelium and soil, yellowish rhizomorphs when immature. Gleba: snuff-brown to ferruginous powdery mass formed by the breakdown of peridioles seated in a small black pocket within the peridium. Peridioles: thin-walled, ovoid-ellipsoid, smooth, clay-buff, 2 × 1 mm, at first clay-buff then more snuff-brown or ferruginous as they mature, discrete, but later breaking down into a snuff brown to ferruginous powdery mass, exuding sienna to fulvous fluid when mounted in alkaline solution.

Constituent hyphae: intertwined, hyaline to ochraceous, thin-walled, 2.4–4.8 mm broad, septate without encrustation, clamp-connections present. Basidia not seen. Basidiospores: globose, 5.8–*9.0*–10.7 µm including spines, heavy ornamentation consisting of isolated groups of connate, narrow cones adhering together to form points, lacking connections.

Additional specimens examined: **THAILAND**, Chiangmai, 11-Aug-2001 (BBH28598; GenBank FR748149), together with other specimens referred to *P. orientalis* (sp. 5) in Table 1.Other sequences obtained were directly from cultures.

The pedicellate, smooth basidiomes that become blackish brown at the disc above the first maturing pseudoperidioles, the relatively small spores that are covered with narrow cones that join together to form connate secondary spines, and an association with *Pinus kesiya* distinguish *Pisolithus orientalis* from other species in the genus. The distinctive spore ornamentation coupled with the molecular data distinguishes this new taxon. The characters emphasis how important it is to examine in detail the development of both basidiome and basidiospores in *Pisolithus*.

Conclusion

Our study confirms that *Pisolithus tinctorius* sensu lato, as previously and widely understood, covers several separate species. Molecular work linked to scanning electron microscope studies of the basidiospores is proving

essential in unravelling speciation within *Pisolithus*. The marriage of data from classical sources and our molecular and morphological studies confirms the distinctiveness of the British, French, and Swedish collections and a new species from SE Asia growing with *Pinus kesiya*.

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